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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :		(11) International Publication Number: WO 92/14824
C12N 15/53, 9/02, A01N 63/00 C12N 15/82, 5/10, A01H 5/00	A1	(43) International Publication Date: 3 September 1992 (03.09.92)

(21) International Application Number: PCT/GB92/00331

(22) International Filing Date: 24 February 1992 (24.02.92)

(30) Priority data:
659,434 25 February 1991 (25.02.91) US

(71) Applicant: IMPERIAL CHEMICAL INDUSTRIES PLC [GB/GB]; Imperial Chemical House, Millbank, London SW1P 3JF (GB).

(72) Inventors: HARTMAN, Christina, L. ; 905 Lois Court, Princeton, NJ 08540 (US). JOHAL, Sarjit, S. ; 15 Teaberry Lane, Newton, PA 18940 (US). SCHMITT, Mark, R. ; 107 Kensington Avenue, Trenton, NJ 08600 (US).

(74) Agent: HUSKISSON, Frank, Mackie; Imperial Chemical Industries plc, Group Legal Department, Patents, P.O. Box 6, Shire Park, Bessemer Road, Welwyn Garden City, Hertfordshire AL7 1HD (GB).

(81) Designated States: AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH (European patent), CI (OAPI patent), CM (OAPI patent), CS, DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GA (OAPI patent), GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU (European patent), MC (European patent), MG, ML (OAPI patent), MN, MR (OAPI patent), MW, NL (European patent), NO, PL, RO, RU, SD, SE (European patent), SN (OAPI patent), TD (OAPI patent), TG (OAPI patent).

Published
With international search report.

(54) Title: NEWLY CHARACTERISED OXALATE OXIDASE AND USES THEREFOR

(57) Abstract

This invention pertains to methods for using oxalate oxidase in plant pathology. A substantially pure gene encoding the oxalate oxidase enzyme is elucidated. The expression product of the gene which can be stably incorporated into a foreign plant host has a unique profile including a pH optimum of 3.5, a positive heat stability, a single sub-unit of approximately 25 kilodaltons and protease stability. The methodology of this invention exploiting oxalate oxidase for protection against oxalic acid encompasses providing to a plant in need of oxalic acid protection an oxalic acid degrading enzyme in an amount sufficient to protect the plant from the oxalic acid.

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NEWLY CHARACTERISED OXALATE OXIDASE AND USES THEREFOR

This invention relates to the genetic improvement of plants by the use of recombinant DNA techniques. Particularly, but not exclusively, the invention relates to the improvement of the tolerance of plants to disease.

From the beginning of agriculture humans have been confronted with the problem of plant disease. Throughout history many strides have been made against plant diseases as exemplified through the use of hybrid plants, pesticides and improved agricultural practices. However, as any farmer, home gardener, or house plant devotee can attest, the problems of plant disease are an ongoing and constant problem in plant cultivation. This invention constitutes a major step forward in solving the problem of plant disease by exploiting a long known fact regarding the method by which certain microbes, especially fungi, attack plants. Specifically, this invention addresses a means by which the etiologic (disease causing) agent is slowed or prevented from actually entering into the plant tissue, thereby preventing disease. Alternatively, in instances where complete invasion is not prevented the present invention confers upon plants a means for reducing the effects of

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infection thereby reducing or preventing plant mortality due to the disease.

In order for a plant pathogen to infect a plant, it must be able to gain access into and subsequently throughout the plant. Plant pathogens accomplish this in various ways. Generally, this is accomplished by the secretion of chemical substances that affect certain component and/or metabolic mechanisms of the plant to be attacked, i.e., the host for the pathogen. The main groups of compounds that are secreted by plant pathogens and that are related to the disease-causing mechanism are toxins, enzymes, polysaccharides and/or other effectors of growth. One such chemical compound is oxalic acid or oxalate which can be degraded by the enzyme oxalate oxidase. Although the reaction catalysed by oxalate oxidase is well-known, the physico-chemical attributes of the enzyme as exemplified by barley oxalate oxidase are incorrectly reported in the literature. The secretion of oxalic acid as a means by which plant pathogens attack plant hosts is commonly found in a plurality of fungal genera and especially in the genera Sclerotinia, Sclerotium, Aspergillus, Streptomyces, Penicillium, Pythium, Paxillus, Mycena, Leucostoma, Rhizoctonia and Schizophyllum. These genera of fungi, especially those fungi of the genus Sclerotinia, are known to cause destructive and fatal diseases of numerous highly cultivated plants including field crops such as sunflower, soybean, beans in general, rape/canola, alfalfa, flax, safflower, peanut and clover, vegetable crops such as lettuce, tomato, cucurbits, potato, carrot, radish, pea, lentils, cabbage,

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broccoli and brussel sprouts, flowers such as petunia and pyrethrum and tree species such as peach. The diseases include not only pre-harvest diseases in the field but also post-harvest diseases during shipping and storage.

The symptoms caused by the aforementioned fungal genera vary with the host plant and the parts of the host plant infected with the disease, as well as being dependent upon environmental conditions at the time of pathogen attack. For example, a feature of all Sclerotinia disease is wilting and collapse of the leaves whereupon the fungus rapidly invades the "heart" of the plant and throughout the stem. The disease is fatal.

In liquid cultures of Sclerotinia levels of oxalic acid ranging from a thousand to ten thousand parts per million, depending upon growth media, age of the culture and other parameters have been observed. Certain plant tissue such as leaves of bean and sunflower exposed to low concentrations of oxalic acid readily show signs of wilting and cell death suggesting the importance of oxalic acid in later stages of disease. The precise mechanism of the disease causing function of oxalic acid after infection is unknown although theories range from chelation of divalent metals interfering with plant cell walls and/or key metabolic enzymes to providing an optimum micro-environment for the action of hydrolytic enzymes secreted by these fungi. Regardless, it is clear from extant evidence that oxalic acid is an integral component of pathogenic attack. Evidence for this conclusion has been obtained by several studies including investigations with oxalate-minus Sclerotinia

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mutants that appear to possess the normal complement of hydrolytic enzymes and other factors but do not produce disease symptomology. Revertants of these oxalate minus mutants demonstrated normal disease development and characteristics.

The high degree of virulence of diseases associated with the aforementioned fungal genera are well-known. For example, leaves of greenhouse grown sunflower plants infected with Sclerotinia sclerotorum frequently exhibit wilting and interveinal necrosis three to five days after inoculation. Study of these plants has shown a wilt inducing substance in water extracts of hypocotyl (the part of the axis of a plant embryo or seedling below the cotyledon) lesions. Chemical tests including thin layer chromatography and gas-liquid chromatography have demonstrated that this wilt inducing substance contained oxalic acid and that wilted leaves from infected plants contained over fifteen times more oxalic acid than leaves of healthy plants. As already noted, the oxalic acid moves systemically through the plant to cause disease symptoms in tissues that are both distant from the initial point of infection and not necessarily infected with fungal hyphae.

With this backdrop, the inventors realised that appropriate identification, isolation and expression of an oxalate degrading enzyme such as an oxalate oxidase, oxalate decarboxylase or similar enzyme by a plant might well diminish the pathogenicity of fungi which secrete oxalic acid as a key component of pathogenicity. Accordingly, the inventors set out and achieved the goal of properly

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identifying and isolating a gene for a protein that is suitable for the introduction of oxalate oxidase activity into plants and microbes using the techniques of genetic engineering.

Oxalates also occur commonly in some plant species as products naturally produced by the plant itself. Naturally-occurring oxalate accumulates in some green leaf vegetables such as spinach and rhubarb and in some forage legumes.

Although such crops species potential sources of dietary vitamins and minerals, ingestion of large amounts is toxic to humans where it binds with calcium ion and precipitates, as insoluble calcium oxalate in the kidneys leading to hyperoxaluria and degradation of renal tissue.

It is also known that oxalic acid may react with other plant metabolites to form oxalate derivatives which are highly toxic. In one example the compound β-N-oxalyl-L-α-β-diaminopropionic acid is formed which is a neurotoxin.

Thus, the presence of oxalic acid in some plant species rules out their use as human or animal food.

In particular, the inventors have characterised an enzyme useful in thwarting pathogenicity involving oxalic acid. This uniquely characterised oxalate oxidase enzyme catalyzes or otherwise contributes to a reaction involving the oxidative degradation of oxalate to produce carbon dioxide and hydrogen peroxide. The general form of this reaction is:

$$\text{Oxalate} + O_2 \xrightarrow[\text{oxidase}]{\text{oxalate}} 2CO_2 + H_2O_2$$

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The study of this enzyme has resulted not only in its identification, isolation and expression, but also in its characterisation and its cloning so that a gene for expression of the enzyme is extant, can be introduced into plants, and expressed by plants thereby conferring disease resistance to fungi in which oxalic acid is a critical component. Such plant transformation would protect the transformed plants against the deleterious disease causing effects of oxalic acid.

It will be appreciated that the applications of the aforementioned inventions are not limited to plant pathogenesis. Still another benefit of this invention is the introduction of an oxalate oxidase gene into a plant to produce a low oxalic acid plant. This could be especially beneficial in high oxalate plants such as peanuts, beets, spinach, rhubarb, barley, cocoa, and many grasses. See, Libert and Franceschi (1987), J/ Agric. Food Chem., 35:926-938. Also, the invention has application for the large scale production of oxalate degrading enzymes. The need for such large scale oxalate degrading enzymes is known in a variety of fields including the need for their use in assay kits to determine the presence and/or amount of oxalic acid and for use in the degradation of oxalic acid present in foodstuffs, beverages and commercial processes. For example, the microbial oxalate decarboxylase has been used by the brewing industry (U.S. Patent No. 4,652,452) and as set forth herein oxalate can also be degraded using oxalate oxidase.

An object of the present invention is to provide means for the degradation of oxalic acid in plants.

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According to the present invention there is provided a cDNA derived from RNA encoding an oxalate oxidase enzyme and having the DNA sequence of Figure 1 herewith, variations thereof permitted by the degeneracy of the genetic code, and genomic DNA to which the said cDNA hybridises.

The invention further provides an enzyme having the ability to degrade oxalate characterised by a single subunit of approximately 25 kilodaltons, and in particular, a substantially pure oxalate oxidase for use in the treatment of plants to degrade oxalic acid therein, said oxalate oxidase having the amino acid sequence shown in Figure 2 and characterised by a pH optimum of 3.5, a positive heat stability, a single subunit of approximately 25 kilodaltons and protease stability.

The said oxalate oxidase may be formulated with an agronomically appropriate carrier for delivery of said oxalate oxidase to a plant.

The present invention, therefore, also provides a method of degrading oxalic acid in a plant comprising delivering to the said plant an oxalic acid degrading enzyme so as to protect said plant from said oxalic acid.

Preferably the enzyme is oxalate oxidase.

Delivery of said oxalic acid degrading enzyme may be effected by applying to the plant or to the locus of the plant said enzyme in combination with an agriculturally acceptable carrier, or, alternatively, by genetic transformation of the plant genome with a gene encoding said oxalate oxidase.

Further the invention provides a method for

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the reduction of oxalate in plant tissue comprising stably incorporating into the genome of the plant a gene encoding the enzyme oxalate oxidase, for example, by genetic transformation.

A suitable gene construct for this purpose may comprise a promoter sequence, a transit peptide, a structural gene sequence encoding said oxalate oxidase enzyme and a gene terminator sequence.

Therefore, the present invention also provides a transformed plant cell comprising a gene for expression of oxalate oxidase. The said gene may have the sequence of Figure 1.

Additionally, the invention provides a method of combating plant pathogenesis by infection with a fungus which secretes oxalic acid, comprising stably incorporating into the genome of said plant by transformation a gene encoding oxalate oxidase.

Examples of fungal genera which secrete oxalic acid are Sclerotinia, Sclerotium, Aspergillus, Streptomyces, Penicillium, Pythium, Paxillus, Mycena, Leucostoma, Rhizoctonia, Whetzelinia and Schizophyllum.

One particular fungus of interest in this invention is Sclerotinia sclerotorum which infects, for example, sunflower (Helianthus annuus)

Thus, in general terms, the present invention is broadly directed to the understanding and appreciation of the use of an oxalate degrading enzyme as exemplified by oxalate oxidase for commercial uses such as in the brewing industry or for agronomic uses such as to reduce susceptibility of a plant to oxalic acid or to reduce the endogenous oxalic acid concentration in a plant.

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The inventors have come to appreciate and teach herein, particularly for agronomic application, the use of oxalate degrading enzymes to reduce plant mortality or destruction from diseases or other phenomenon in which oxalic acid plays a critical invasive role. The inventors have also appreciated that use of this invention can result in the prevention of plant mortality and infection from diseases in which oxalic acid is critical. Such diseases are particularly caused by, among others, the specific genera of fungi set forth herein.

The appreciation of the inventor's novel uses of oxalate degrading enzymes is especially enhanced by the realisation and invention of the inventors that the physico-chemical properties of barley oxalate oxidase as reported in the literature was inaccurate. Accordingly, the inventors have substantially purified and characterised on oxalate oxidase enzyme not heretofore described. Indeed, the purported substantial purifications of the enzyme as set forth in the literature are shown herein to be incorrect. Specifically, the inventors disclose a substantially pure oxalate oxidase having a particular total amino acid composition, shown in Figure 2, a pH optimum of 3.5, a neutral isoelectric point, a positive heat stability, protease stability and a single subunit of approximately 25 kilodaltons.

Also set forth herein is the invention of substantially all of a substantially pure gene encoding an oxalate oxidase enzyme with a specific DNA sequence as shown in Figure 1. The gene encodes the oxalate oxidase enzyme having the characteristics as set forth above. In particular

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the gene encodes an enzyme exhibiting oxalate oxidase activity having a single subunit of approximately 25 kilodaltons, that reacts specifically with antibodies raised against purified barley oxalate oxidase, and has an amino acid sequence shown in Figure 2.

The invention also discloses compounds for use in combating plant pathogenesis which compounds include chemicals exhibiting oxalic acid degrading activity in particular oxalate oxidase activity. Specifically, the plant compound has oxalate oxidase activity in an amount sufficient to break down oxalic acid produced by pathogens. It will be appreciated that another agronomic use for such a compound is to combine the compounds with an appropriate carrier, which is agronomically acceptable, permitting delivery of the compound directly to the plant or to the soil.

A transformed plant cell is also disclosed herein, which cell is transformed by a gene encoding the expression of oxalate oxidase or another oxalate degrading enzyme. The gene encoding such enzyme can include the DNA sequence set forth in Figure 1, which substantially corresponds to the oxalate oxidase enzyme substantially purified by the inventors.

A method is disclosed herein for providing protection against oxalic acid to a plant in need of protection from such oxalic acid. The method includes providing an oxalic acid degrading enzyme in an amount sufficient to protect the plant from the oxalic acid to a plant in need of such protection. Preferably, it is envisioned that the oxalic acid degrading enzyme is oxalate oxidase

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encoded by a gene having the structure shown in Figure 1.

The methodology for providing such protection can take a plurality of forms including the transformation of a plant with a gene encoding an oxalic acid degrading enzyme and in particular encoding oxalate oxidase. Alternatively, the method can include provision of an oxalic acid degrading enzyme in combination with an agronomically acceptable carrier for direct application to a plant or to soil in which the plant grows.

The purified oxalate oxidase of this invention, its use as an agent to fight pathogenesis and its use in plant cell transformation provide an innovative and unique approach to the control of plant diseases in which oxalic acid plays a critical component either during pathogenesis or at the invasive stage. It is, of course, well-known that the activity of the enzyme oxalate oxidase is that it contributes to the degradation of oxalic acid. However, it was the current inventors who first appreciated that by attacking the oxalic acid by chemical degradation, for example by enzymatic degradation, a significant agricultural benefit in conferring disease resistance against those diseases in which oxalic acid plays a critical role would result. This invention holds special promise because a major scourge in the commercial cultivation of agronomically important plants, for example crops such as sunflowers, is caused by fungal species such as Sclerotinia that secrete oxalic acid.

The benefits of the inventor's insight can be

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exploited either by plant transformation or by application of oxalate oxidase as a traditional pesticide most probably in combination with a suitable carrier that is agriculturally acceptable. One of the important benefits of the use of oxalate oxidase as a pesticide is that it is ecologically sound, non-polluting and does not harm the plant.

If an external application of the enzyme is to be used to protect a plant or plant part against pathogens, it would be expected that the enzyme would be diluted to form a liquid solution or suspension or mixed with a diluent solid to be applied as a dust. The precise nature of application will depend in part on the particular pathogen(s) and plant(s) targeted. Detailed methods for adapting general methods of application to specific crops and pathogens can be found in "Methods for evaluating pesticides for control of plant pathogens". K.D. Hicky, ed., The American Phytopathological Society, 1986. Adjuncts that could be added to the formulation include agents to aid solubilization, wetting agents and stabilisers, or agents that would produce a microencapsulated product. Such adjuncts are well-known in the art.

External applications could also utilise recombinant micro-organisms in either a viable form or after being converted into a non-viable form by a method that does not inactivate the enzyme.

Although the prior art reportedly purified oxalate oxidase and characterised it, the inventors have discovered that such literature reports were inaccurate and that the enzyme was, in fact, never properly purified and characterised. It will be appreciated that as used herein, "oxalate oxidase"

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refers to the purified and characterised form of the enzyme as set forth herein unless stated otherwise. The differences between the extant literature reports and the purified and characterised enzyme of the current invention are set forth in detail herein and can be briefly gleaned from Table 1.

The impurity of commercially available oxalate oxidase preparations as well as the improper characterisation of the enzyme in the literature was evident when the inventors first purified oxalate oxidase from barley seedling root. Two procedures were used. In one instance purification of the barley seedling root oxalate oxidase involved homogenisation of frozen tissue in 1 to 4 volumes of water and purification from the aqueous extract following filtration through cheesecloth to remove debris. The solution was further purified by centrifugation at 18,000 g for 30 minutes followed by heat treatment at 80°C for 3 minutes with the precipitates at both steps being discarded; protein precipitation from the supernatant between 30% and 70% saturation with respect to ammonium sulphate $(NH_4)_2SO_4$ was collected by centrifugation and was dialyzed against water. Protein resolubilizing from the ammonium sulphate precipitation step was fractionated on a Pharmacia FPLC using a Mono S 10/10 column equilibrated with 25 mM potassium acetate pH 4.8 eluted with a 0.0 to 0.4 M NaCl gradient in the same buffer. Oxalate oxidase activity was measured by the method of Sugiura, et al (1979), Chem. Pharma. Bull. 27(9):2003. Peak fractions of oxalate oxidase activity were

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combined, equilibrated with the low salt potassium acetate pH 4.8 buffer and re-chromatographed on the FPLC using a Mono S 5/5 column eluted with the buffers and NaCl gradient as above. Peak fractions from the Mono S 5/5 step were combined, equilibrated with 25 mM Tris-Cl, pH 7.6 applied to a Mono Q 5/5 column and eluted with a 0.0 to 0.4 m NaCl gradient. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of peak oxalate oxidase activity fractions showed prominent protein bands upon silver staining at approximately 25 and 38-40 kilodaltons.

Subsequent size fractionated of the native protein on a Superose-12 gel filtration column (equilibrated with 50 mM potassium acetate, pH 4.8) on the FPLC showed a well-defined peak of activity eluting at a time corresponding with the molecular weight of about 25,000. No oxalate oxidase activity was found eluting in any other fractions associated with other molecular weights.

A second method involved the use of detergent extraction. Four to seven day old barley seedling roots were powdered in the presence of liquid nitrogen and stored at -80°C. Storage under these conditions resulted in no apparent loss of activity. The stored tissue was homogenised with distilled water containing 0.5% taurodeoxycholate sodium salt, filtered and centrifuged. Oxalate oxidase assays on the two fractions (supernatant and pellet) reveal that both fractions possessed activity. Accordingly, the pellet was extracted with distilled water containing 0.5% taurodeoxycholate. Following exhaustive dialysis against distilled water, ammonium sulphate was

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added to concentrate and fractionate the soluble supernatant probe. The precipitated proteins (30-70% ammonium sulphate fractions) were then resuspended in a small volume of distilled water containing detergent and desalted on a small gel permeation column (Sephadex G25). The active fraction was applied to an anion exchange column (DEAE) using a Tris-HCl, pH 7.5 buffer and elution of bound protein effected using a sodium chloride gradient. The enzymatically active fraction was concentrated, desalted and then applied to a Mono-Q column (Pharmacia). A sodium chloride gradient was again employed to elute the proteins. Activity assays showed that the oxalate oxidase was concentrated in 3 fractions. Upon analysis of these fractions by SDS polyacrylamide gel electrophoresis, the activity was determined to be associated with a polypeptide of about 25 kilodalton molecular weight.

Following purification, characterisation of the purified, homogeneous oxalate oxidase preparation was effectuated. As already indicated and as shown in Table 1 the properties of the protein resulting from this purification plainly differ from that described in the literature. This suggests that despite attempts by others skilled in the art to purify the oxalate oxidase enzyme, it was not until the present efforts of the inventors that this enzyme was properly described and fingerprinted through the elucidation of its physico-chemical properties. The results of the physico-chemical characterisation undertaken by the inventors are set forth in Table 1. By way of comparison, the previously reported literature

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characterisation of the enzyme is also set forth.

TABLE 1

OXALATE OXIDASE PROPERTIES

Property	Literature	Invention
pH Opt.	3.5	3.5
Heat Stab.	+	+
Subunit No.	2	1
Size	75k	25k
pI	2.8	Approx 7
Confactors	-	-
Protease Sta	Unknown	+
Memb. Assoc.	Unclear	+

Further establishing the distinctiveness of the inventors' enzyme exhibiting oxalate oxidase activity can be seen in Table 2. As Table 2 plainly evidences this invention establishes an amino acid composition different than reported in the literature. See, Chiriboga, J. (1966) Archives of Biochemistry and Biophysics 116, 516-523.

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TABLE 2

Amino Acid	Invention	Literature
Asx	12.53	8.44
Ser	8.65	6.50
Gly	13.31	10.75
Glx	7.42	6.80
Thr	8.10	5.18
Ala	7.93	7.85
Val	8.41	6.40
Met	2.85	0
Tyr	0.98	0.93
Ile	2.15	6.39
Leu	9.36	-
Phe	6.89	-
His	1.79	1.68
Lys	5.98	4.90
Trp	-	-
Arg	3.65	3.48

Partially purified barley seed root oxalate oxidase was solubilized, subjected to cation exchange chromatography using a Mono S column (25 mM potassium acetate, pH 4.8, and eluted with a 0 to 400 millimolar potassium chloride gradient in the same buffer). The recovered enzymatically active protein was further purified by preparative sodium dodecyl sulphate polyacrylamide gel electrophoresis. A single protein band of about 25 kilodalton molecular weight band was detected and subsequently sliced from the gel.

The wet gel slices were fragmented, mixed with Freund's adjuvant and injected intramuscularly into the rear of the thigh muscle near the hip of the

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rabbits. The rabbits were boosted with additional quantities of protein and sample bleeds monitored to assure the production of oxalate oxidase specific antibodies. After about four months the animals were exsanguinated. The polyclonal rabbit antibodies produced were of very high titer.

The gene encoding for barley-derived oxalate oxidase having the structure shown in Figure 1, was cloned as shown in Figure 4 using a cDNA library constructed from barley root. Total RNA was prepared from barley root. Polyadenylated RNA was then recovered from total RNA and used for the synthesis of cDNA. Both total and polyadenylated RNA were prepared using commercially available RNA extraction and mRNA purification kits according to the standard instructions provided with the kits (Pharmacia LKB Biotechnology Inc). Construction of the library was commercially performed (Clontech Laboratories). The vector chosen for the library was lambda gt 22. Use of this expression vector was selected based on the high titer and specificity of the oxalate oxidase antiserum. Whatever the expression vector selected for use with this conversion, it is advantageous for the vector to permit the use of an immunological screen for the protein product of oxalate oxidase cDNA clones. Base on the average insert size of 1.8 kilobases for the cDNA library and good representation as far as number of independent clones (1.7×10^6), it was deduced that the library should contain a cDNA clone for the oxalate oxidase protein approximately 25 kilodaltons in size.

The initial screen of the library was performed with antiserum to oxalate oxidase

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according to standard procedures as, for example, set forth in Huynh, T., et al., (1985) DNA Cloning Techniques: A Practical Approach, D. Glover, ed. IRL Press, Oxford. Since information on the N-terminal amino acid sequence of the mature oxalate oxidase protein had been obtained (except for the N-terminal residue), the N-terminal sequence was also used to confirm the identity of any cDNA clones recovered as positive in the initial immunological screen. 1.2×10^6 plaques from the cDNA library were screened with the antiserum. Fourteen potentially positive signals were obtained one of which was much stronger than the others. Two sequential rounds of re-screening with the antiserum were performed on these fourteen clones in order to obtain confirmed positive, single plaque isolates which would be characterised at the molecular level. Plaque number 12 again gave the strongest signal throughout these subsequent screens. Plaque number 12 was then used to purify the oxalate oxidase antibody as a test of the specificity of the plaque signal, according to the standard procedure as described, for example, in Hunyh, T. et al., supra. Antibody purified from plaque 12 was used as a probe on a Western blot of an acrylamide gel of oxalate oxidase protein. The plaque-purified antibody reacted specifically with oxalate oxidase, showing a pattern identical to that seen with purified antibody as a probe.

The insert (cDNA product cloned into the vector lambda gt 22) from plaque 12 was recovered using standard polymerase chain reaction (PCR) methods as exemplified in Ausubel, F.M. et al., eds., (1988). "The Polymerase Chain Reaction", in

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Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, New York, pp. 15.0.1-15.4.6. The insert in plaque number 12 was estimated to be 650-750 base pairs. The size of the insert in plaque 12 was consistent with a protein of about 25 kd. Based on the initial size estimate for the cDNA insert and the result from plaque purification of the antibody, plaque number 12 was considered to be the best candidate for further molecular analysis. The number 12 insert prepared by PCR was then recloned into a plasmid vector more amenable to analysis. The cDNA insert from clone 12 was also used as a probe to a Northern blot of barley root RNA to estimate the size of the mRNA for oxalate oxidase. Formaldehyde gel electrophoresis and transfer to a nylon membrane were carried out according to the procedures recommended by the commercial supplier of the membranes, Schleicher and Schuell. The number 12 probe hybridised to a single mRNA species approximately 800-850 bases long.

Dideoxy nucleotide sequencing was performed on the recloned plaque 12 insert. A commercially available T7 sequencing kit (Pharmacia LKB Biotechnology Inc.) and the manufacturer's recommended procedure were used. The barley cDNA insert was determined to be approximately 690 bases long with some uncertainty in two small regions covering no more than ten base pairs each, the c terminus and the identity of the three N terminal bases. The nucleotide sequence of the clone 12 insert is shown schematically in Figure 1. The partial protein sequence was then predicted from the nucleotide sequence of clone 12 and compared to

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the N-terminal amino acid sequence obtained directly from the purified oxalate oxidase. This comparison is shown in Figure 3.

The gene having the structure shown in Figure 1 containing the coding sequence for the mature oxalate oxidase enzyme would be attached to genetic regulatory elements which are needed for the expression of the structural gene in a defined host cell. The first type of regulatory element required is a gene promoter region, which contains DNA sequences recognised by the biological machinery of the plant cell and which induces transcription of the DNA sequence into messenger RNA (mRNA). The mRNA is then translated into the protein produce codes for by the structural gene region. The promoter is attached in front of or 5′ to the gene for oxalate oxidase, which can be performed according to standard methods know in the art. See, for example, T Maniatis, et al., (1982) Molecular Cloning, Cold Spring Harbor Laboratory, New York, pp. 104-106. Promoter regions which could be used for expression of the oxalate oxidase gene in plant cells include promoters which are active in a wide range of different plant tissues. For example, the 35S promoter from the cauliflower mosaic virus may be suitable for this purpose. Another type of promoter that could be used in plant cells is one which expresses under more restricted conditions. Included in this class would be promoters active only in certain tissue(s) of the plant and/or induced to be active by certain stimuli like wounding. An example of this kind of promoter would be the 5′ regulatory region from the gene for phenylalanine ammonia lyase (PAL). This

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type of promoter is discussed in Liang, X. et al., (1989), PNAS, USA, 8:9284-9288. Expression of the oxalate oxidase gene in microbial hosts could be achieved by use of promoters obtained from microbial sources. Examples of such promoters would include the trp promoter for expression in bacteria such as E. Coli as exemplified in Amann, E., et al., (1983) Gene, 25: 167-178, or the glyceraldehyde phosphate dehydrogenase (GAPD) promoter for expression in yeast as exemplified in Edens, L. et al, (1984), "Synthesis and Processing of the Plant Protein Thaumatin in Yeast," Cell 37:629-633. The gene promoter sequences may also be derived in part or in whole from promoter sequences found in cells unlike those of the host cell as long as they meet the above criteria for transcription and translation.

A second genetic regulatory element which desirably could be, but need not be, attached to the oxalate oxidase gene is a terminator or polyadenylation sequence which promotes effective termination of transcription of the gene and, in eukaryotes, also promotes polyadenylation, ie, the addition of any number of adenosine nucleotides at the 3′ end of the mRNA. Standard methods known in the art can be used to attach the terminator region behind or 3′ to the gene. (See, for example, T. Maniatis, et al, supra, pp. 104-106). An example of such a terminator/polyadenylation sequence for expression in plants would be that from the octopine synthase gene from an Agrobacterium tumefaciens Ti plasmid as enuciated in H. DeGreve et al., (1982), "Nucleotide sequence and transcript map of the Agrobacterium tumefaciens Ti

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plasmid-encoded octopine synthase gene", J. Mol. Appl. Genet., 1:499-511. An example of such a terminator for expression in microbial hosts is the rho-independent transcription terminator sequence from Salmonella typhimurium. See, for example, M.E. Winkler, (1987), "Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology", F.C. Neidhardt, ed-in-chief; American Society for Microbiology. The gene terminator sequences may also be derived in part or in whole from terminator sequences found in cells unlike those of the host cell, as long as they meet the above criteria for transcription termination and polyadenylation required by the host cell.

Another type of regulatory element which may be attached to the gene for oxalate oxidase is a DNA sequence coding for a signal peptide. The signal peptide is attached to the amino terminus of the protein and permits the protein to be localised to the cell wall or secreted from the host cell. During this localisation process, the signal peptide is cleaved off, producing a protein product with the sequence of the mature protein. The DNA sequence for the signal peptide is inserted between the promoter and the coding region. Standard methods known in the art may be used to attach the DNA sequence for the signal peptide (See, for example, Maniatis, T., et al., supra, pp. 104-106). Examples of such signal sequences would include the signal peptide from an extension gene of plants (Chen, J. and Varner, J.E., an extracellular matrix protein in plants: characterization of a genomic clone for carrot extension". EMBO J 4:2145-2151, 1985) from the bacterial pelB (pectate lyase) gene

of Erwinia carotovora (Lei, S.P. et al., (1987), J. Bacteriol. 169 4379) and from perpro factor of yeast (Smith, R.A., et al., Science 229:1219-1229, 1985). The signal peptide sequences may also be derived in whole or in part from terminator sequences found in cells unlike those of the host cell, as long as they meet the above criteria for processing and localisation of the protein in the host cell.

Any of the various methods known for introducing foreign genes into plants could be used for insertion of the oxalate oxidase gene into a host plant. The methodology chosen to accomplish plant transformation with the oxalate oxidase gene would vary depending on the host plant. By way of example, one well-characterized methodology that would be useful for plant transformation with the oxalate oxidase gene would be Agrobacterium mediated transformation.

Agrobacterium mediated transformation using the oxidase gene follows the procedures well-known for this methodology. First, a gene cassette suitable for expression in plants is introduced into a disarmed strain of Agrobacterium tumefaciens as an intermediate host. The oxalate oxidase gene cassette is introduced into the T-DNA region of a recombinant plasmid containing a selectable marker gene such as a gene encoding for neomycin phosphotransferese II, phosphinothricin acetyl transferase or the like. This methodology is set forth in many literature publications including Horsch, et al., (1985),. "A Simple and General Method for Transferring Genes Into Plants", Science 227:1229-1231. Pieces of plant tissue, eg leaf,

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cotyledon or hypocotyl are co-incubated with the bacteria for 2-3 days before the bacteria are killed using antibiotics such as carbenicillin. Additional antibiotics corresponding to the selectable marker gene employed are included in the plant tissue culture medium such that only transformed plant cells will grow.

Plants regenerated from the transformed cells are then tested for the presence and expression of the oxalate oxidase gene. Immuno assays and test for oxalate oxidase activity can be used to identify individual transformants. Tolerance to exogenous oxalic acid can also be used as a functional test of intact tissues.

As noted, several other methodologies are available for plant transformation apart from Agrobacterium transformation. Examples of these other DMA delivery methods include electroporation, ie chemically induced delivery into protoplasts, micro-injected, biolostics as well as others. An example of a type of plant that is not especially suitable for Agrobacterium-mediated transformation are soybean and certain cereals including maize. These plants would plainly benefit from plant transformation attempts using methodologies other than Agrobacterium-mediated transformation.

The drawings which accompany this description are as follows:

Figure 1 shows a cDNA sequence reverse engineered from the mRNA coding for barley seedling oxalate oxidase.

Figure 2 shows the amino acid sequence translated from the cDNA shown in Figure 1.

Figure 3 shows diagrammatically the cloning

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strategy used to clone the oxalate oxidase gene and described in detail in Examples 1 and 2 hereinbelow.

Figure 4 shows diagrammatically the cloning strategy described in Examples 3 and 4 below.

Figure 5 shows the structure of plasmid pVB1, and,

Figure 6 shows the structure of plasmid pBin19i.

The invention will now be described, by way of illustration, in the following Examples.

EXAMPLE 1

Construction of an oxalate oxidase non-secretion vector for tomato transformation.

(a) Reconstruction of a full length oxalate oxidase open reading frame (ORF).

Oligonucleotides 1219901A and 1219901A(compl) were designed and synthesised to form a linker with a HindIII site at the 5′ end and a BsaI site at the 3′ end.

oligo 1219901A:

5′-AGCTTGATGGGTACCTCGGACCCAGACCCACTCCAGGACTTCTGCGTC GCGGACCTCGATGGCAAGGCGGTCTCG-3′

OLIGO 1219901A(COMPL)

5′-TCACCGAGACCGCCTTGCCATCGAGGTCCGCGACGCAGAAGTCCTGGA GTGGGTCTGGGTCCGAGGTACCCATCA-3′

The oligonucleotides were annealed and cloned into clone #22 cut with HindIII and partially with BsaI to produce the clone pOXOX1 containing the mature oxalate oxidase ORF as indicated by peptide sequencing.

After cloning of the HindIII/BsaI linked into clone #22, the integrity of the mature oxalate oxidase ORF was confirmed by DNA sequencing.

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(b) Insertion of the oxalate oxidase ORF into an expression vector.

The mature oxalate oxidase coding region was isolated from agarose as a 700 bp HindIII/BamHI fragment and the ends were made blunt with T_4 DNA polymerase. pNB33A was cut with XbaI and PstI, the vector fragment was isolated from agarose and the ends made blunt with T_4 polymerase.

These two blunt ended fragments were ligated together to produce the clone pNBOXOX1. The correct orientation of the oxalate oxidase coding region in pNBOXOX1 was confirmed by DNA sequencing.

(c) Transfer of the oxalate oxidase expression cassette into pBin19Ri.

pBNOXOX1 was cut with HindIII and partially with EcoRI. The 1.9 kb expression cassette was isolated from agarose and cloned into pBin19Ri (Figure 6) cut with EcoRI and HindIII to give clone pNOX1.

The presence of the oxalate oxidase expression cassette in clone pNOX1 was confirmed by restriction of plasmid DNA with EcoRI and HindIII.

EXAMPLE 2

Construction of an oxalate oxidase non-secretion vector for sunflower transformation.

(a) Transfer of the oxalate oxidase expression cassette into pVB1.

The 2.1 kb EcoRI/HindIII fragment containing the oxalate oxidase expression cassette isolated from pNBOXOX1, described in Example 1 above, was cloned into pVB1 (Figure 5) cut with EcoRI and HindIII to give clone pVBOX1.

The presence of the oxalate oxidase expression

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cassette in clone pVBOX1 was confirmed by restriction of plasmid DNA with EcoRI and HindIII.

(b) Insertion of the Hygromycin resistance gene into pVBOX1.

A 2.3 kb HindIII fragment containing the Hygromycin resistance cassette was isolated from p19HYG and cloned into pVBOX1 cur with HindIII to give clone pHOX1.

The presence and orientation of the Hygromycin cassette in clone pHOX1 was confirmed by digestion of plasmid DNA with EcoRI.

EXAMPLE 3

Construction of an oxalate oxidase secretion vector for tomato transformation.

(a) Production of an extensin transit peptide/oxalate oxidase fusion by PCR.

In order to fuse the mature oxalate oxidase to an extensin transit peptide, oligonucleotides EXTOX-5 and EXTOX-3 were designed and synthesised.

EXTOX-5

5'-TTCAACTGGTACCATTTGTTTCAAAGATGGGAAAAATGGCTTCTCTAT TTGCCACATTTTTAGTGGTTTTAGTGTCACTTAGCTTAGCTTCTGAAACCG ACCCAGACCCACTCCAGGACTTC-3'

EXTOX-3

5'-CTATTAAATTCGCGGTACCTGGATATATAGAATTAC-3'

The oligonucleotides were used as PCR primers with pNBOX1 as template. The resulting 771bp EXTOX PCR product containing a 23 amino acid extensin transit peptide fused in frame to the mature oxalate oxidase coding region was cut with KpnI.

(b) Insertion of the EXTOX PCR product into pJR1(pUC).

The KpnI digested EXTOX PCR product described above was cloned into pJR1(pUC) cut with KpnI to

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give the clone pJEXTOX1. The orientation and sequence of the EXTOX PCR product in pJEXTOX1 was confirmed by DNA sequencing.

(c) Transfer of the EXTOX expression cassette into pBin19i.

The extensin/oxalate oxidase expression cassette was isolated from pJEXTOX1 as a 1.5 kb EcoRI/HindIII fragment and cloned into pBin19i cut with EcoRI and HindIII to produce the clone pNEXTOX1.

The presence of the expression cassette in pNEXTOX1 was confirmed by digestion of plasmid DNA with EcoRI and HindIII.

EXAMPLE 4

Construction of an oxalate oxidase secretion vector for sunflower transformation.

(a) Isolation of the secretion expression cassette and insertion into pVB1.

The extensin/oxalate oxidase expression cassette was isolated from pJEXTOX1 as a 1.5 kb EcoRI/HindIII fragment and cloned into pVB1 cut with EcoRI and HindIII to produced the clone pVEXTOX1.

The presence of the expression cassette in pVEXTOX1 was confirmed by digestion of plasmid DNA with EcoRI and HindIII.

(b) Insertion of the Hygromycin resistance gene into pVEXTOX1.

A 2.3 kb HindIII fragment containing the Hygromycin resistance cassette was isolated from p19HYG and cloned into pVEXTOX1 cur with HindIII to give clone pHEXTOX1. The presence and orientation of the Hygromycin cassette in clone pHEXTOX1 was confirmed by digestion of plasmid DNA with EcoRI.

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CLAIMS

1. A cDNA derived from RNA encoding an oxalate oxidase enzyme and having the DNA sequence of Figure 1, and variations thereof permitted by the degeneracy of the genetic code.

2. A genomic DNA to which the cDNA claimed in claim 1 hybridises.

3. An enzyme having the ability to degrade oxalate characterised by a single subunit of approximately 25 kilodaltons.

4. A substantially pure oxalate oxidase for use in the treatment of plants to degrade oxalic acid therein, said oxalate oxidase having the amino acid sequence shown in Figure 2 and characterised by a pH optimum of 3.5, a positive heat stability, a single subunit of approximately 25 kilodaltons and protease stability.

5. The oxalate oxidase claimed in claim 4, further comprising an agronomically appropriate carrier for delivery of said oxalate oxidase to a plant.

6. A method of degrading oxalic acid in a plant comprising delivering to the said plant an oxalic acid degrading enzyme so as to protect said plant from said oxalic acid.

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7. The method of claim 6 wherein said enzyme is oxalate oxidase.

8. The method of claim 6 wherein delivery of said oxalic acid degrading enzyme is effected by applying to the plant or to the locus of the plant said enzyme in combination with an agriculturally acceptable carrier.

9. The method of claim 7 wherein the said oxalate oxidase enzyme is delivered to the plant by genetic transformation of the plant genome with a gene encoding said oxalate oxidase.

10. A method for the reduction of oxalate in plant tissue comprising stably incorporating into the genome of the plant a gene encoding the enzyme oxalate oxidase.

11. A method as claimed in claim 10, wherein the said gene in incorporated into the plant by genetic transformation.

12. A method as claimed in claim 11 in which the plant genome is transformed by a gene construct having a promoter sequence, a transit peptide, a structural gene sequence encoding said oxalate oxidase enzyme and a gene terminator sequence.

13. A transformed plant cell comprising a gene for expression of oxalate oxidase.

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14. The transformed cell of claim 13 wherein said gene includes the DNA sequence of Figure 1.

15. The transformed cell of claim 13 wherein said oxalate oxidase expressed by said gene is a single subunit of approximately 25 kilodaltons.

16. A method of combating plant pathogenesis by infection with a fungus which secretes oxalic acid, comprising stably incorporating into the genome of said plant by transformation a gene encoding oxalate oxidase.

17. A method as claimed in claim 16 in which the fungus is of a genus selected from the group of oxalic acid secreting fungi comprising Sclerotinia, Sclerotium, Aspergillus, Streptomyces, Penicillium, Pythium, Paxillus, Mycena, Leucostoma, Rhizoctonia, Whetzelinia and Schizophyllum.

18. A method as claimed in claim 17 in which the Sclerotinia sclerotorum.

19. A method as claimed in claim 16 in which the plant is sunflower (Helianthus annuus)

20. A plant having stably incorporated within its genome by transformation a gene encoding oxalate oxidase.

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BNSDOCID: <WO__9214824A1_L>

SEQ ID NO: 1

FIG. 1

SEQUENCE TYPE: Nucleotide

SEQUENCE LENGTH: 702bp

STRANDEDNESS: Single

TOPOLOGY: Linear

MOLECULE TYPE: DNA

ORIGINAL SOURCE ORGANISM: Barley

IMMEDIATE EXPERIMENTAL SOURCE: pGEM11Zf(+) clone #22

FEATURES:
From 1 to 603: region coding oxalate oxidase

PROPERTIES: fragment of cDNA coding oxalate oxidase

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FIG. 1 cont.

```
GACCCAGACC CACTCCAGGA CTTCTGCGTC GCGGACCTCG ATGGCAAGGC GGTCTCGGTG  60
AACGGGCATA CGTGTAAGCC CATGTCGGAG GCCGGCGACG ACTTCCTCTT CTCGTCCAAG 120
CTGACCAAGG CCGGCAACAC GTCCACCCCG AACGGCTCGG CCGTGACGGA GCTCGACGTG 180
GCCGAGTGGC CCGGTACGAA CACGCTGGGC GTGTCCATGA ACCGTGTGGA CTTCGCGCCG 240
GGGGGCACCA ACCCGCCGCA CATCCACCCG CGTGCAACCG AGATCGGCAT GGTGATGAAA 300
GGTGAGCTCC TCGTTGGAAT CCTCGGCAGC CTTGACTCCG GAAACAAGCT CTACTCCAGG 360
GTGGTGCGTG CCGGAGAGAC TTTCGTCATC CCGCGCGGCC TCATGCACTT CCAGTTCAAC 420
GTTGGTAAGA CGGAAGCCTA CATGGTTGTG TCCTTCAACA GCCAGAACCC TGGCATCGTC 480
TTCGTGCCGC TCACACTCTT CGGCTCCGAC CCTCCCATCC CCACGCCCGT GCTCACCAAG 540
GCTCTCCGGG TGGAGGCCGG AGTCGTGGAA CTTCTCAAGT CCAAGTTCGC CGGTGGGTCT 600
TAACTTCCAT GAGCCCCAAA TGATCAATAT GAATATGTAA TTCTATATAT CCATGTATGC 660
TGCGAATTTA ATAGTACTCG ACAGGAGACT ATACCGGAAT TC                      702
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FIG. 2

Translation of Sequence ID No: 1

	5					10					15					20					25					30				
1	D	P	D	P	L	Q	D	F	C	V	A	D	L	D	G	K	A	V	S	V	N	G	H	T	C	K	P	M	S	E
31	A	G	D	D	F	L	F	S	S	K	L	T	K	A	G	N	T	S	T	P	N	G	S	A	V	T	E	L	D	V
61	A	E	W	P	G	T	N	T	L	G	V	S	M	N	R	V	D	F	A	P	G	G	T	N	P	P	H	I	H	P
91	R	A	T	E	I	G	M	V	M	K	G	E	L	L	V	G	I	L	G	S	L	D	S	G	N	K	L	Y	S	R
121	V	V	R	A	G	E	T	F	V	I	P	R	G	L	M	H	F	Q	F	N	V	G	K	T	E	A	Y	M	V	V
151	S	F	N	S	Q	N	P	G	I	V	F	V	P	L	T	L	F	G	S	D	P	P	I	P	T	P	V	L	T	K
181	A	L	R	V	E	A	G	V	V	E	L	L	K	S	K	F	A	G	G	S										

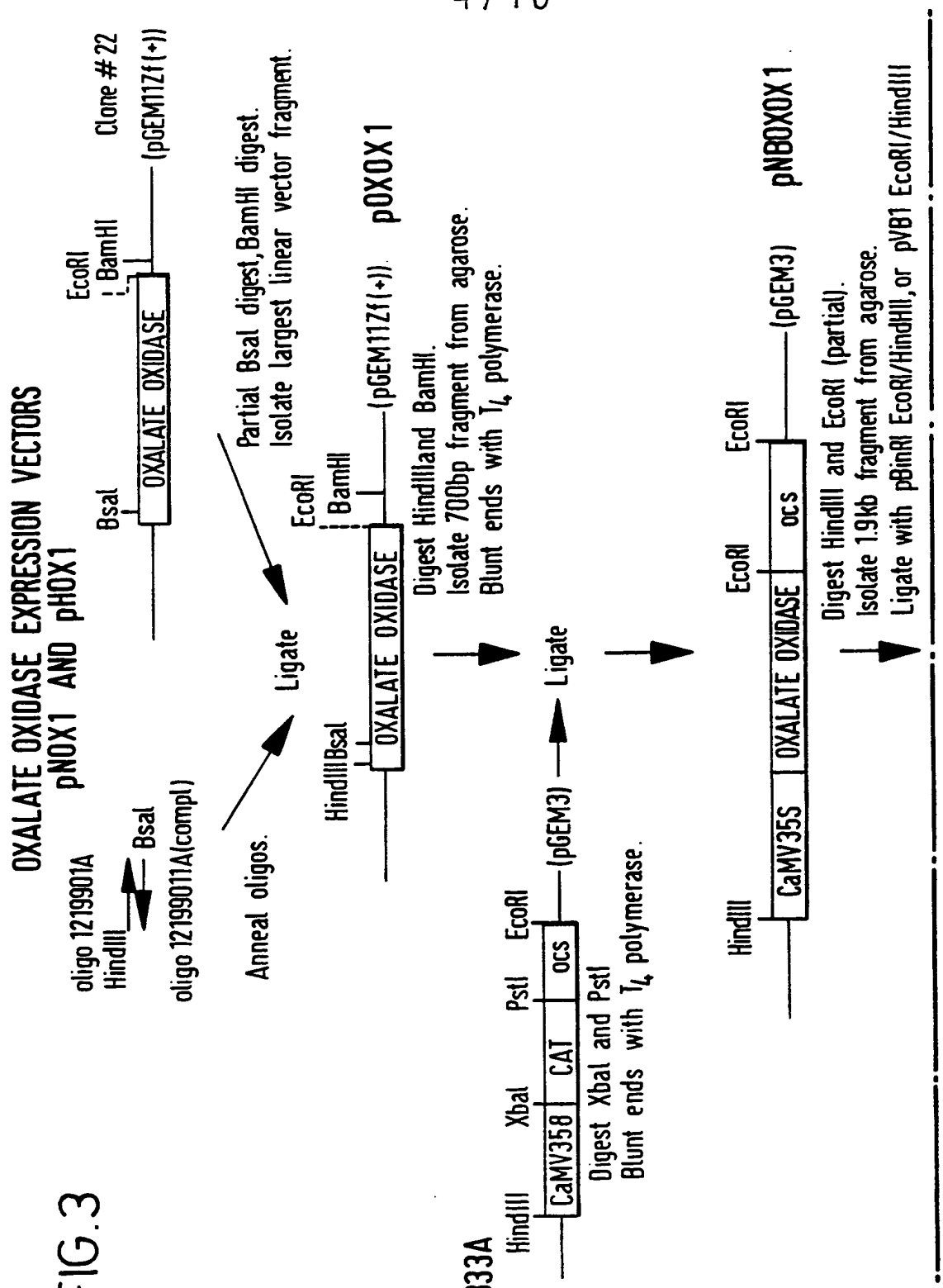

OXALATE OXIDASE EXPRESSION VECTORS
pNOX1 AND pHOX1
oligo 1219901A
HindIII
BsaI
oligo 12199011A(compl)
Anneal oligos.
Ligate
BsaI
OXALATE OXIDASE
EcoRI
BamHI
Clone #22
(pGEM11Zf(+))
Partial BsaI digest, BamHI digest.
Isolate largest linear vector fragment.
HindIIIBsaI
OXALATE OXIDASE
EcoRI
BamHI
(pGEM11Zf(+))
pOXOX1
Digest HindIIIand BamHI.
Isolate 700bp fragment from agarose.
Blunt ends with T4 polymerase.
pNB33A
HindIII
XbaI
PstI
EcoRI
CaMV35S
CAT
ocs
(pGEM3)
Digest XbaI and PstI
Blunt ends with T4 polymerase.
Ligate
HindIII
CaMV35S
OXALATE OXIDASE
ocs
EcoRI
EcoRI
(pGEM3)
pNBOXOX1
Digest HindIII and EcoRI (partial).
Isolate 1.9kb fragment from agarose.
Ligate with pBinRI EcoRI/HindIII, or pVB1 EcoRI/HindIII

FIG.3

1/2

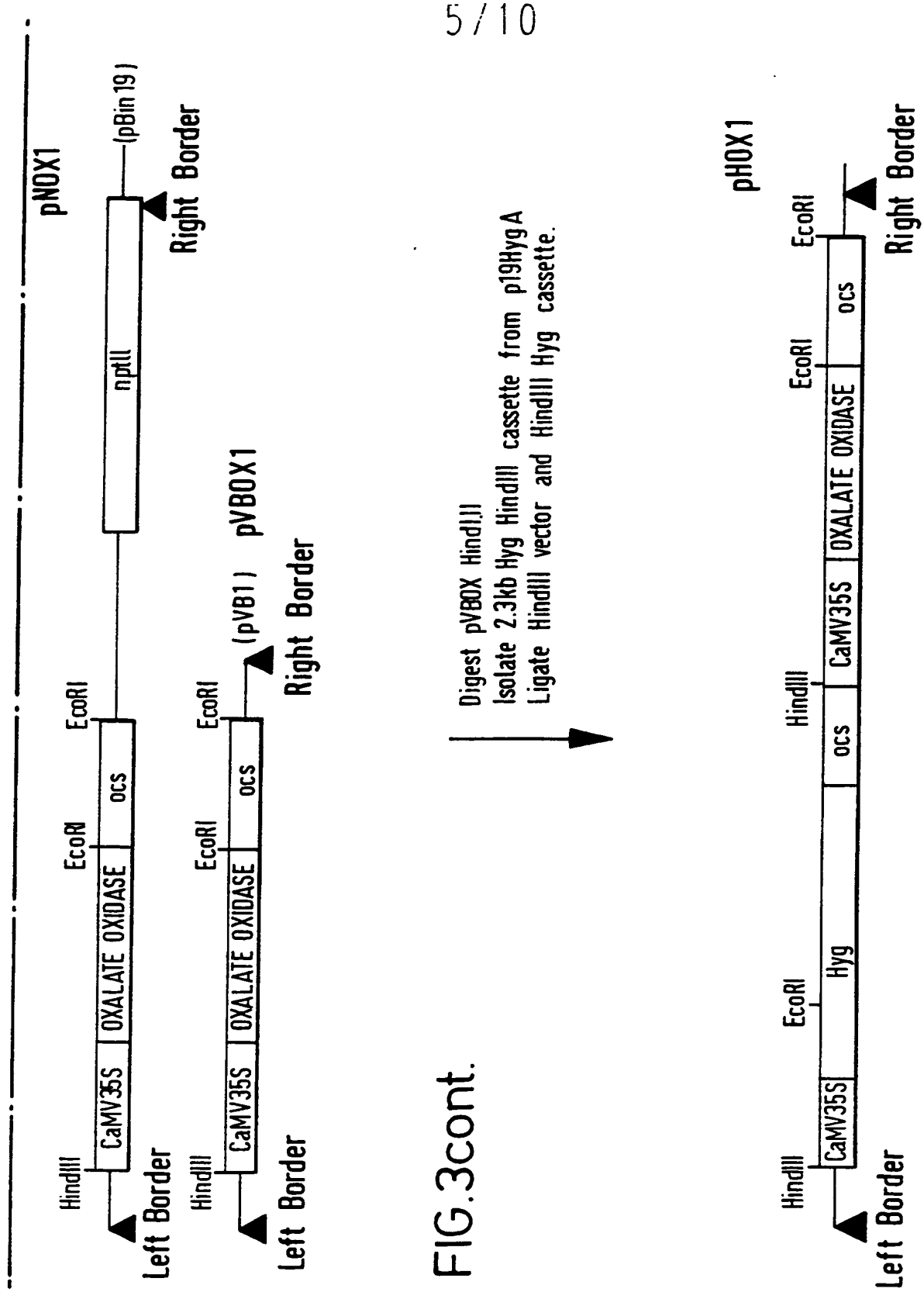
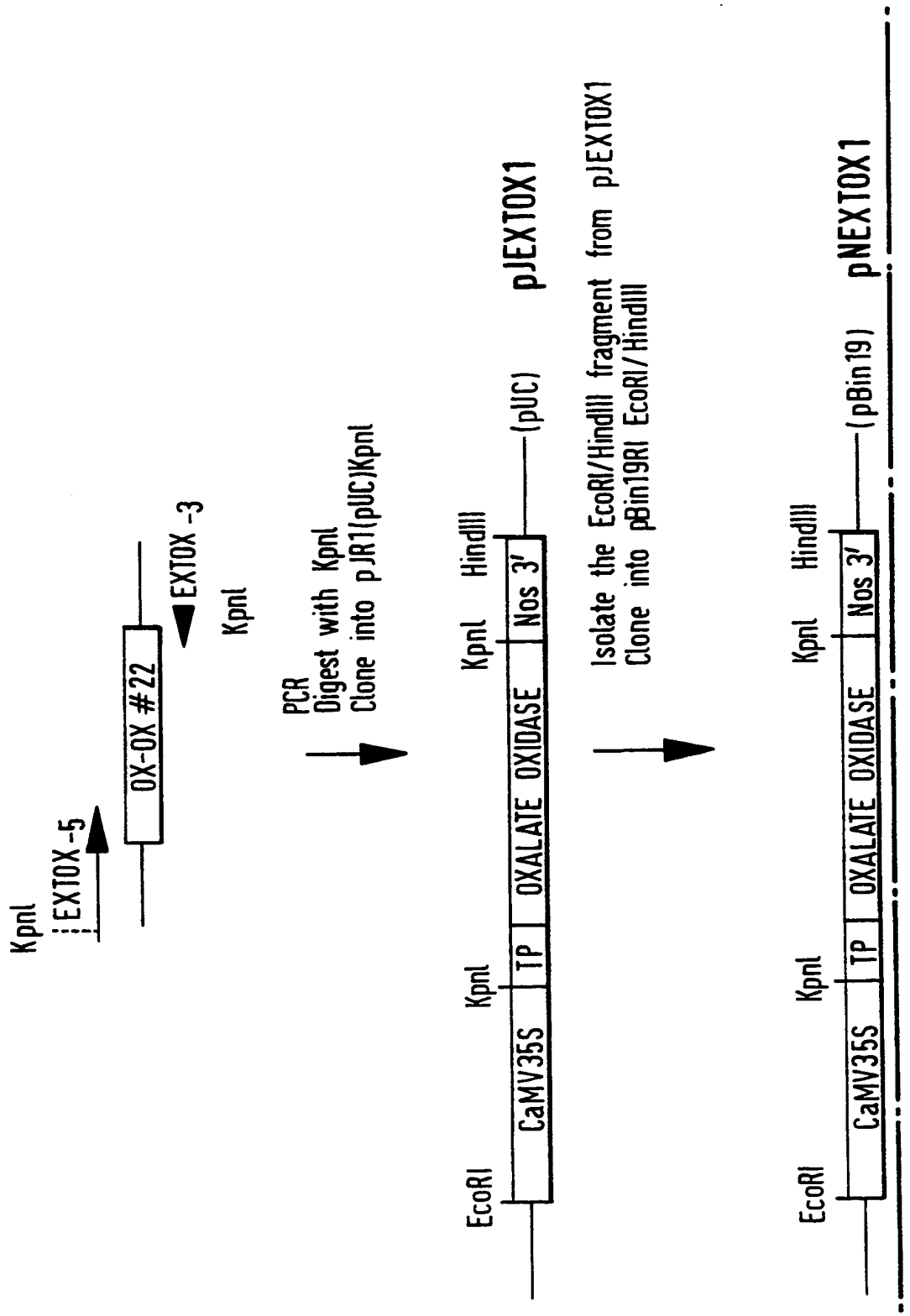

pNOX1
(pBin 19)
Right Border
nptII
EcoRI
EcoRI
CaMV35S
OXALATE OXIDASE
ocs
EcoRI
HindIII
Left Border
(pVB1)
pVBOX1
Right Border
EcoRI
EcoRI
CaMV35S
OXALATE OXIDASE
ocs
HindIII
Left Border
Digest pVBOX HindIII
Isolate 2.3kb Hyg HindIII cassette from p19HygA
Ligate HindIII vector and HindIII Hyg cassette.
pHOX1
Right Border
EcoRI
EcoRI
CaMV35S
OXALATE OXIDASE
ocs
HindIII
EcoRI
Hyg
ocs
HindIII
Left Border

FIG.3cont.

FIG. 4



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FIG. 4 cont.

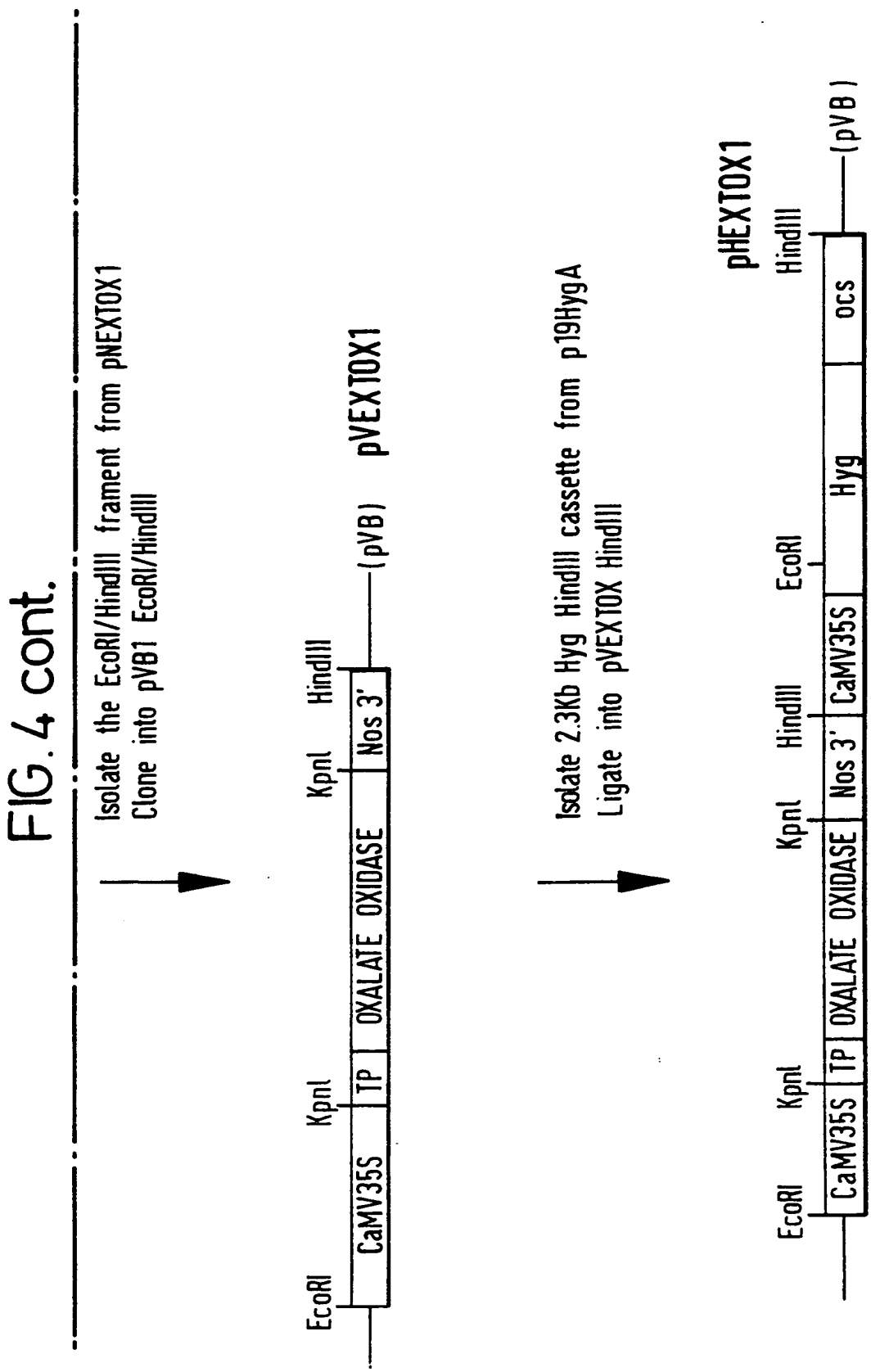

Isolate the EcoRI/HindIII frament from pNEXTOX1
Clone into pVB1 EcoRI/HindIII
pVEXTOX1
(pVB)
EcoRI
KpnI
CaMV35S
TP
OXALATE OXIDASE
KpnI
HindIII
Nos 3'
Isolate 2.3Kb Hyg HindIII cassette from p19HygA
Ligate into pVEXTOX HindIII
pHEXTOX1
(pVB)
EcoRI
KpnI
CaMV35S
TP
OXALATE OXIDASE
KpnI
HindIII
Nos 3'
CaMV35S
EcoRI
Hyg
HindIII
ocs

FIG.5

pVB1 = Transformation vector base

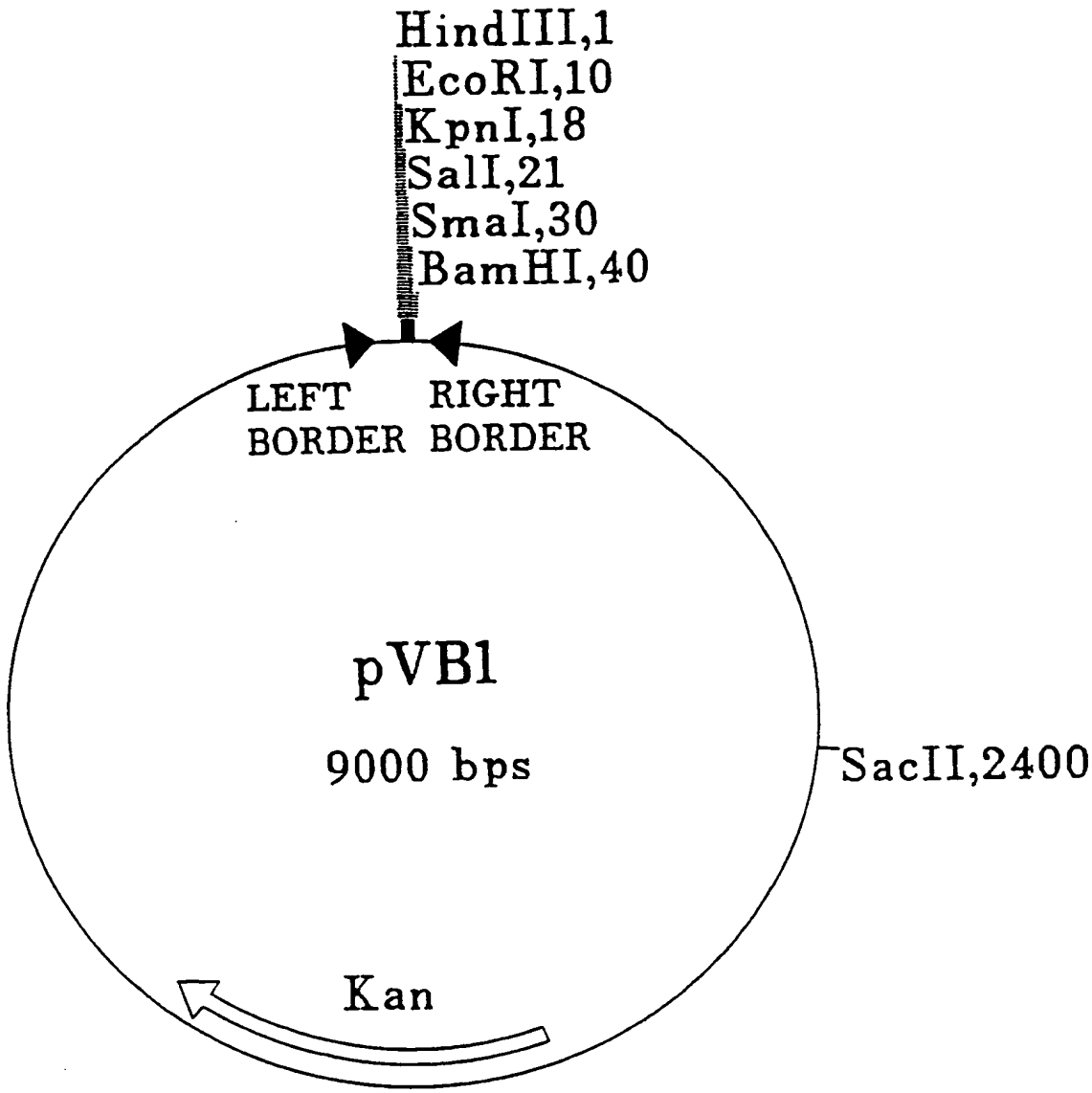
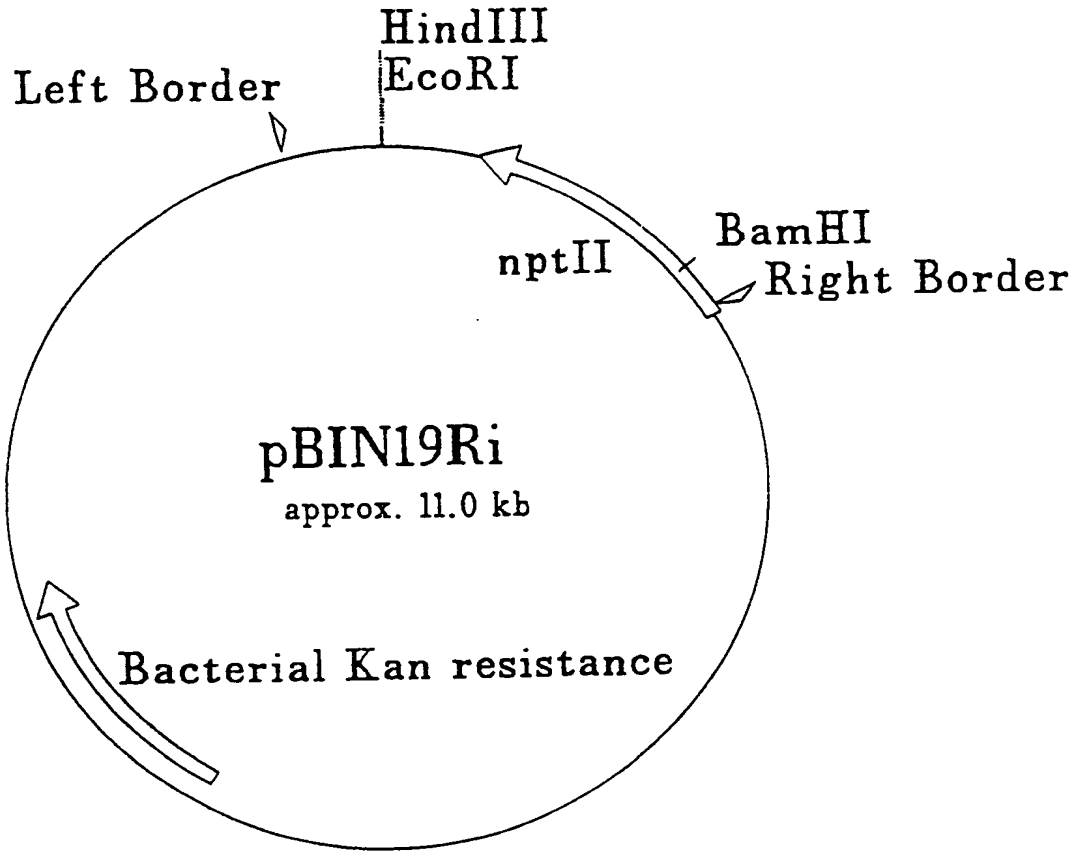


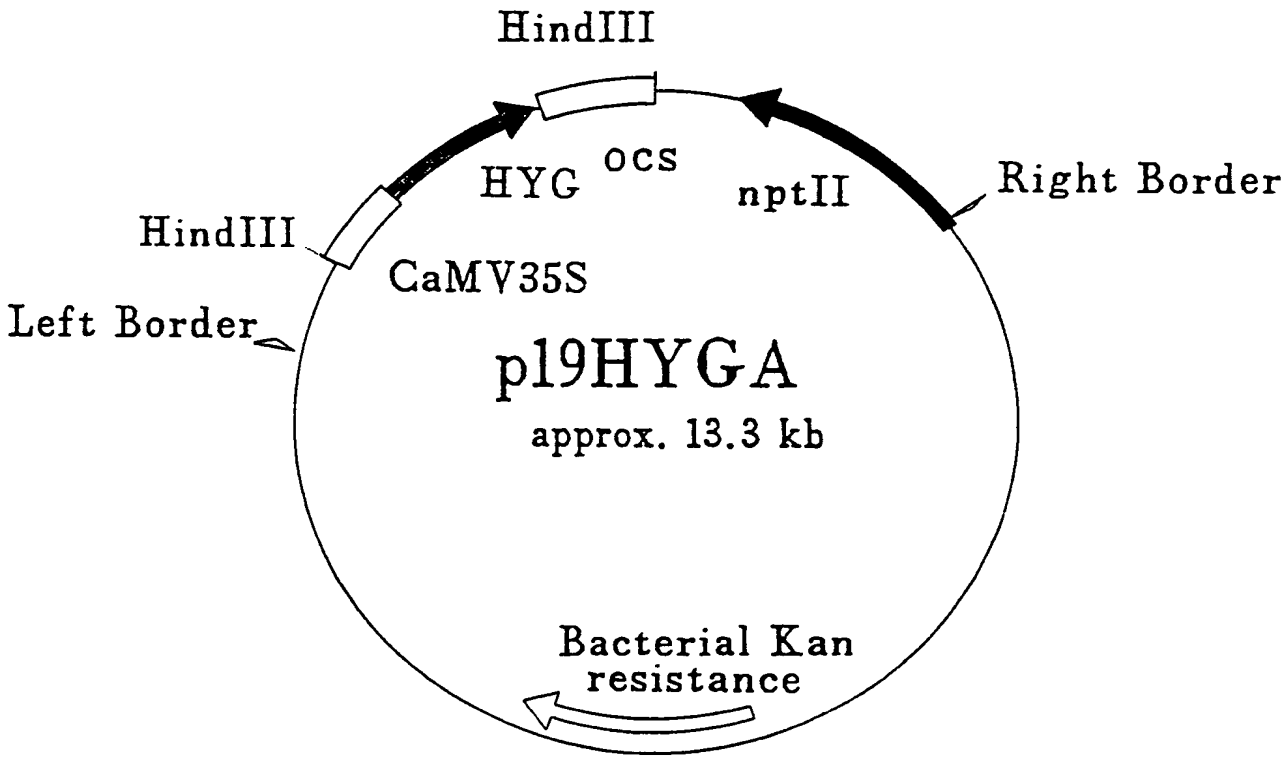
FIG.6
Structure of pBIN19Ri



pBIN19Ri contains the wild type nptII gene. The orientation of the EcoRI and HindIII sites in pBIN19Ri is reversed with respect to the parent plasmid pBIN19 (M. Bevan, NAR vol 12, 8711-8721 (1984)).

FIG. 7

Structure of p19HYGA



CaMV35S = Cauliflower mosaic virus 35S promoter
ocs = Octopine synthase 3' region
Parent plasmid is pBIN19.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 92/00331

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/53; C12N9/02; A01N63/00; C12N15/82
C12N5/10; A01H5/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; A01N ; A01H

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 264, no. 9, 25 March 1989, BALTIMORE US pages 4896 - 4900; DRATEWKA-KOS, E., ET AL.: 'Polypeptide structure of germin as deduced from cDNA sequencing' see figure 2 ---	1-3
O,P, X	PLANT PHYSIOLOGY. vol. 96, no. 1, 1991, ROCKVILLE, MD, USA. page 85; SCHMITT, M. R., ET AL.: 'Barley seedling oxalate oxidase purification and properties' see abstract 540 --- -/--	3,4

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
22 MAY 1992	03. 06. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MADDOX A.D.

Form PCT/ISA/210 (second sheet) (January 1985)

BNSDOCID: <WO___9214824A1_I_>

International Appli[illegible] No

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECO[illegible]HEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	AGRICULTURAL AND BIOLOGICAL CHEMISTRY. vol. 52, no. 3, 1988, TOKYO JP pages 743 - 748; KOYAMA, H.: 'Purification and characterization of oxalate oxidase from Pseudomonas sp. OX-53' see page 743, left-column see page 747, left column ---	1-4
A	WO,A,8 808 450 (FINLAYSON) 3 November 1988 see page 17 - page 22 see page 67 - page 68; claim 8 ---	1-4
A	CHEMICAL ABSTRACTS, vol. 98, 1983, Columbus, Ohio, US; abstract no. 13533, see abstract & PREP. BIOCHEM. vol. 12, no. 4, 1982, pages 341 - 353; PIETTA, P. G., ET AL.: 'Improved purification protocol for oxalate oxidase from barley roots' ---	1-4

BNSDOCID: <WO___9214824A1_I_>

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. GB 9200331
SA 56776

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BNSDOCID: <WO___9214824A1_I_>